DESCRIPTION

RECOMBINANT PROTEIN POSSESSING HUMAN HEPATITIS C VIRUS RNA-DEPENDENT POLYMERASE ACTIVITY AND METHOD OF PRODUCING THE SAME

TECHNICAL FIELD

The present invention relates to a soluble recombinant protein possessing hepatitis C virus RNA-dependent RNA polymerase activity.

BACKGROUND ART

Hepatitis C virus (HCV) is a + positive-strand RNA virus that was isolated and identified as the causative virus for non-A non-B hepatitis by a group of Chiron in 1989. Etiologic surveys have resulted in the estimation that about 1% of the world population is infected with HCV. Important routes of infection remain unknown except for those via blood, such as blood transfusion; the contribution of vertical infection from parents to children is assumed to be minor. A new immunological diagnostic method for HCV developed by Chiron has been used with dramatically successful results in the prevention of HCV infection via blood transfusion.

Despite its identity as an RNA virus, HCV causes chronically persisting infection in the liver, which can lead to chronic hepatitis. HCV-induced chronic hepatitis persisting for more than about 30 years represents the major cause of subsequent hepatoma (liver cancer); in Japan, at least 75% of all liver cancer cases develop in the presence of HCV-induced hepatitis as an underlying disease.

HCV-associated liver cancer develops after a characteristic series

of events such that the pathogenic risk can be predicted from the course of pathologic state. It is therefore expected that blocking HCV virus proliferation would mitigate hepatitis by HCV and effectively prevent the progress or development of liver cancer.

Although various approaches are being investigated in an attempt to prevent HCV infection and proliferation, no decisive methods have been established.

First, immunological studies with HCV surface proteins as targets for the prevention of viral infection have failed to yield desired achievements, due to the potential of the viral surface proteins for sequential variations in the course of infection, nor has anyone succeeded in identifying receptors required for HCV infection on the host side.

Moreover, the viral protein, synthesized as a single long polypeptide (see FIG. 1), is cleaved by proteases encoded by the genes of the host and HCV during its synthesis, resulting in the production of the structural and nonstructural proteins of the virus. Among these proteases are those encoded by HCV NS2 and NS3; there have been studies with NS2 and NS3 in an attempt to inhibit the protein cleavage process by these proteases, and structural analyses and development of inhibitors are ongoing; however, much more time will be taken to clinical application.

The lagging in the research into the HCV proliferation process and the development of HCV vaccine is mainly attributable to the absence of an HCV infection/proliferation system using cultured cells; however, since an infectious HCV RNA clone has recently been identified in an experimental system using the chimpanzee, it is expected that establishing an HCV proliferation system in cultured cells would

accelerate the production of attenuated virus, the development of vaccine, the elucidation of the molecular mechanism of the HCV proliferation process, and also the development of proliferation inhibitors. In short, development of an HCV proliferation/replication system would enable the elucidation of the HCV replication process and its molecular mechanism, and hence the designing of a proliferation inhibitor based on the specificity of HCV proliferation. Currently, however, relevant research activities fail to directly meet the great and urgent demand.

DISCLOSURE OF THE INVENTION

In addition to the above-described conventional approaches, it would be possible to contribute to the treatment and prophylaxis of HCV infection by inhibiting the RNA-dependent RNA polypeptide (hereinafter referred to as RdRP) activity of NS5B, the key enzyme involved in HCV replication (see FIG. 1). Specifically, although HCV is an RNA virus, host does not have a process for synthesizing nucleic acids (genetic information), wherein RNA is synthesized from RNA. For this reason, substances that specifically inhibit the process of HCV replication by RdRP are under development. To obtain such substances, however, a large amount of purified NS5B protein is necessary to investigate RdRP function and assay candidate inhibitors. For this purpose, the method in which an appropriate host is allowed to produce a recombinant NS5B protein possessing RdRP activity by gene recombination technology is the best; however, purified NS5B has not been obtained in sufficient amounts to date.

For example, De Francesco *et al.* obtained recombinant NS5B using insect cells and biochemically demonstrated RdRP activity but

failed to obtain the enzyme in sufficient amounts to assay and develop inhibitors (De Francesco, R., *et al.*, Methods Enzymol. 275:58-67, 1996). Also, many researchers have attempted to express and purify recombinant NS5B possessing RdRP activity in large amount using *Escherichia coli* systems but obtained nothing more than insoluble preparations, which must be denatured and reconstituted, failing to obtain the NS5B protein retaining the essential biochemical activity in sufficient amounts (Tan, B.E., *et al.*, Biochem. Biophys. Res. Commun. 1997).

The present inventors have found that the NS5B protein having the RNA-dependent RNA polymerase (hereinafter, referred to as "RdRP) activity of human hepatitis C virus has an anchor region at the C-terminal region, and investigated its role. The inventors then found that when NS5Bt, which had been obtained by deleting a given number of C-terminal amino acids from NS5B while maintaining the activity, is expressed in a host cell (*e.g.*, *Escherichia coli*) as a fusion protein with another polypeptide, a soluble recombinant NS5Bt protein with the RdRP activity can be obtained, and established the present invention. Thus, NS5B is present in an insoluble fraction in the protein purification process because of the presence of anchor region. However, the NS5Bt protein lacking the anchor region being present in a soluble fraction while maintaining the RdRP activity, it is now possible to recover and purify the recombinant polypeptide from the transformants while maintaining the RdRP activity (see, Test Example 2, Fig. 10).

The present invention provides a method of producing a recombinant RNA-dependent RNA polymerase, comprising transforming a host cell with an expression vector containing both a DNA encoding a polypeptide possessing polymerase activity derived from human

hepatitis C virus RNA-dependent RNA polymerase and a DNA encoding a second polypeptide other than said first polypeptide (*e.g.*, glutathione S-transferase, hereinafter referred to as GST), culturing the transformant obtained, recovering a fusion protein possessing polymerase activity from the medium, and, if desired, separating and recovering the polypeptide possessing polymerase activity from said fusion protein, and also provides a soluble recombinant fusion or non-fusion protein possessing RdRP activity produced by said method.

The method of the present invention has made it possible to provide soluble purified recombinant NS5B protein with RdRP activity in large amounts for the first time, as shown in Examples below.

The soluble polypeptide derived from human hepatitis C virus RdRP of the present invention is normally a polypeptide having a C-terminal region, including the entire or portion of the anchor region of RdRP, deleted.

Since human hepatitis C virus NS5B possesses RdRP activity in the HCV genomic DNA, the term "NS5B" is used herein as having the same definition as that for the "RdRP" of human hepatitis C virus. Also, the soluble polypeptide (protein) possessing RdRP activity, and having 1 or more amino acids on the C-terminal side deleted, is referred to as NS5Bt. It should be noted that the terms "NS5B" and "NS5Bt" also indicate the DNAs encoding the NS5B and NS5Bt proteins, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing the structures of the HCV polyprotein precursor and NS5B protein thereof. The upper panel is a schematic diagram showing the structure of the HCV polyprotein precursor; the middle panel is the hydrophobicity profile of the NS5B

protein (Kyte and Doolittle). The anchor region deduced using the SOSUI program is shaded. The lower panel is a schematic diagram showing the structures of various GST-NS5B fusion protein expression constructs. Each amino acid number in the figure corresponds to that on the HCV polyprotein precursor. NS5B has all amino acids of NS5B; NS5Bt has the 21 amino acids (2989-3010) deleted; NS5Bt-m1 has the GDD motif substituted by VDD; NS5Bt-m2 and NS5Bt-m3 have a relative basal residue cluster substituted by alanine; NS5Bt-m4 has a mutation in the putative anchor region.

FIG. 2 is a restriction map for the GST expression plasmid pGENKS used for the expression of a fusion protein with GST. The plasmid pGENKS encodes the consensus sequence of protein kinase A, thrombin cleavage site and additional multiple cloning sites (*Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sal*I and *Bam*HI) downstream from GST-encoding DNA.

FIG. 3 is a schematic diagram of the construction of the plasmid pGENKS. The site of cleavage by thrombin is underlined. The nucleotide sequence of the DNA and the amino acid sequence encoded thereby are shown in SEQ ID NO: 3.

FIG. 4 shows diagrams for the various processes of purification of GST-NS5Bt as expressed in *Escherichia coli*; panel A shows the results of separation by SDS-1% PAGE of the GST-NS5Bt obtained from a culture, and subsequent staining by Coomassie staining (CBB). Lane 1 shows the results for the whole cell extract; lane 2 shows the results for the centrifugal supernatant of the sonication product; lane 3 shows the results for the cell-free extract after passage through DEAE Sephacel; lane 4 shows the results for the eluate from the column of glutathione Sepharose 4B; lane 5 shows the results for the eluate from the column of poly(U) Sepharose; lane 6 shows the results for non-fusion type

rNS5Bt after thrombin treatment. Panels B and C show the results of western blotting using the anti-NS5B antibody and the anti-GST antibody, respectively.

FIG. 5 shows the results of western blotting analysis of purified GST-NS5Bt protein corresponding to the 95 kDa protein in FIG. 4A, lane 5, using serum obtained from a chronic hepatitis patient infected with HCV 1b or a healthy volunteer. In FIG. 5, lanes 1 and 2 are the purified GST-NS5Bt and the GST protein as control, respectively. The arrow indicates the 95 kDa.

FIG. 6 shows graphs of the RdRP activity of purified GST-NS5Bt in UMP incorporation assay. Panel A is a graph showing the substrate specificity to [α-^{32}P]UMP or [α-^{32}P]dTTP with poly(A) or poly(dA) as a template and oligo$(U)_{14}$ or oligo(dT) as a primer. Panel B shows the time courses of reactions at 25℃ and 37℃; panel C is a graph showing the relationship to the amount of GST-NS5Bt.

FIG. 7 shows graphs of the RdRP activity of purified GST-NS5Bt, based on UMP incorporation, determined under various reaction conditions. In the figure, panel A shows the relationship of UMP incorporation to pH; panel B shows the relationship to temperature; panel C shows the relationship to KCl concentration.

FIG. 8 shows graphs of the RdRP activity of purified GST-NS5Bt, based on UMP incorporation, determined under various reaction conditions. In the figure, panel D shows the relationship of UMP incorporation to Mg^{2+} ion concentration; panel E shows the relationship to Zn^{2+} concentration (0, 10, 25 and 50 μM).

FIG. 9 shows diagrams of the results of RNA synthesis assay using RNA transcribed *in vitro*. Panel A is a construction scheme for the RNA template; panel B is an electrophoretic migration pattern obtained

by reacting at 30℃ for 2 hours using an RNA(s) transcribed *in vitro* as a template and a primer, extracting the RNA product with an organic solvent followed by ethanol precipitation, and separating and purifying the precipitates with 8% PAGE containing 8 M urea.

FIG. 10 shows the photomicrographs showing the subcellular localization of NS5Bt expressed in mammalian cells. Panels (A) through (D) give the results of fluorescent microscopic observation of the subcellular localization of the expression product in HLE cells transfected with expression plasmids encoding (A) full-length NS5B, (B) GFP-NS5Bt, (C) GFP-NS5B-m4, and (D) GFP alone, respectively.

THE BEST EMBODIMENT FOR PRACTICING THE INVENTION

Regarding the genome encoding the HCV protein, cDNA cloning has resulted in the demonstration of the genomic composition. For example, the HCV-JK1 cDNA encodes the 3010 polyprotein precursor, as shown in FIG. 1, which precursor is processed by viral and host proteases to give at least 10 products. Among the 10 products is NS5B, which is closest to the C-terminus and which consists of 591 amino acids; NS5B is thought to encode RdRP polymerase, judging from its sequence in comparison with other viruses (Honda, M., S. Kaneko, M. Unoura, K. Kobayashi, and S. Murakami, Arch Virol. 128:163-169, 1993).

The base sequence and putative amino acid sequence of a DNA encoding such NS5B, which consists of 591 amino acids, in the HCV-JK1 cDNA, are shown by SEQ ID Nos. 1 and 2 in the sequence Listings. The nucleotide sequences in the specification and drawings are conveniently written with capital letters, which correspond to the sequences of the same SEQ. ID. No. written with small letters in the

Sequence Listings provided below.

In the Examples given herein, recombinant NS5Bt possessing RdRP activity was produced using the HCV-JK1 cDNA NS5B as a starting material; however, as long as the object of the present invention is accomplished, any optionally chosen RdRP-encoding NS5B derived from HCV can be used to produce the active recombinant NS5Bt protein in the same manner as described herein. Specifically, the anchor region can be specified from the amino acid sequence of the desired HCV-derived NS5B when the sequence is known; when the sequence is unknown, the anchor region can specified on the basis of the sequence after the sequence is determined by a conventional method. The number of amino acids to be deleted is chosen as appropriate, according to the HCV protein from which the NS5B is derived. Next, a DNA encoding the NS5Bt (1st polypeptide) whose amino acid sequence has been determined is prepared by synthetic or another appropriate means, an expression vector encoding a fusion protein with an appropriate second polypeptide is constructed, a host cell is transformed with the vector, the resulting transformant is cultured, and the active product is recovered. Such a method is obvious to those skilled in the art.

The second polypeptide mentioned herein may be any one, as long as it is expressed as a fusion protein possessing RdRP activity without adversely affecting the RdRP activity when a DNA encoding it is transformed into an appropriate host cell together with another DNA encoding the desired NS5Bt. It is preferable, however, that the second polypeptide possess properties advantageous for the purification and recovery of the fusion protein, with greater preference given to polypeptides permitting the separation of NS5Bt from the fusion protein.

Such polypeptides include glutathione S-transferase (GST), which is preferred because of the ease of obtainment of an expression vector having multiple cloning sites, the possibility of affinity purification and other aspects.

Accordingly, in an embodiment of the present invention, the soluble polypeptide derived from human hepatitis C virus RdRP has either the sequence represented by amino acid Nos. 1 through 570 in the amino acid sequence shown by SEQ ID NO:1 or an amino acid sequence resulting from the deletion, substitution or addition of 1 or more amino acids in said sequence, and possesses polymerase activity.

The position for introduction of amino acid deletion, substitution or addition, the kinds and number of amino acids, etc. can be determined by those skilled in the art on the basis of the sequences and activity determination methods disclosed herein.

The recombinant RdRP of the present invention is novel and useful in the prophylaxis and treatment of hepatitis C. The present invention accordingly provides a DNA encoding said recombinant RdRP, an expression vector containing said DNA, and a transformant resulting from transformation using said expression vector.

The present invention is hereinafter described in detail with reference to HCV-JK1 cDNA-derived NS5B and GST.

In the present specification, the NS5B protein derived from an HCV-JK1 cDNA and the NS5Bt protein prepared therefrom may be simply referred to as NS5B and NS5Bt, respectively. Also, the fusion protein of NS5Bt and glutathione S-transferase (hereinafter referred to as GST) may be referred to as GST-NS5Bt, and the recombinant NS5Bt protein produced by the method of the present invention may be referred to as rNS5Bt or NS5Bt.

In addition, for the purpose of the present invention, the terms "expression vector" and "expression plasmid" are used as mutually exchangeable.

As stated above, the HCV-JK1 cDNA encodes the 3010 polyprotein precursor; NS5B, which encodes RdRP, consists of 591 amino acids closest to the C-terminus (FIG. 1). HCV genomic cDNA analyses by the present inventors have demonstrated that a highly hydrophobic region (aa570-586 (corresponding to 2989-3005 of the entire sequence)) is present at the C-terminus of NS5B which consists of 591 amino acids in the sequence shown by SEQ ID NO:1, suggesting that the anchor region is located around the 21 amino acids on the C-terminal region (Nos. 571-591 (2990-3010) in SEQ ID NO:1) (shaded portion in the middle panel of FIG. 1). With this in mind, the present inventors attempted to express in host cells a fusion protein of a variant (NS5Bt) having some amino acids deleted from this anchor region and GST by gene recombinant technology, and succeeded in obtaining the soluble NS5Bt protein of the fusion or non-fusion type which possesses RdRP activity very efficiently.

A DNA encoding NS5B, which consists of 591 amino acids, and the DNA encoding NS5Bt, which has a given number of amino acids deleted from the C-terminus side, can be synthesized, or obtained from a known HCV-JK1 cDNA by restriction enzyme cleavage, PCR, etc.

Regarding the number of amino acids to be deleted from NS5B, shown by SEQ ID NO:1, preference is given to polypeptides having a given number of amino acids deleted from the region from amino acid No. 591 to amino acid No. 571 as counted from the C-terminus. Examples of such polypeptides include polypeptides having 21 amino acids deleted.

Also, for the purpose of the present invention, not only the C-terminus-deleted polypeptide derived from SEQ ID NO:1 (NS5Bt) but also variants of said polypeptide having an amino acid sequence variation, and possessing RdRP activity, are also useful, provided that the desired RdRP activity is exhibited. Such variants can, for example, be derived by introducing amino acid deletion, substitution and/or insertion to the polypeptide of the amino acid sequence consisting of amino acid Nos. 1 through 570 by a method known to those skilled in the art. Such deletions and additions include amino acid deletions and/or additions at the N- and/or C-terminus. The resultant variants can be screened according to a method known in the art or that shown in Reference Example 2 below.

A DNA encoding NS5Bt as obtained by an optionally chosen method and another DNA encoding GST as the second polypeptide are ligated together in an appropriate expression vector to construct a fusion protein expression vector. Such an expression vector can be constructed by inserting the NS5Bt DNA and the GST DNA, previously ligated together, into an appropriate expression vector, or, as described in the Examples below, by inserting the NS5Bt DNA into an appropriate GST expression vector [*e.g.*, pGENK1 (Murakami, S. *et al.*, J. Biol. Chem. 269:15118-15123, 1994; Yi, M.-K. *et al.*, Virology 231:119-129, 1997] so that the DNA encoding GST and NS5Bt is expressed as a fusion protein. The plasmid pGENKS, described later, encodes the consensus kination site of protein kinase A, thrombin cleavage site and additional multiple cloning sites (*Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sal*I and *Bam*HI). This pGENKS vector is suited for the purpose of the present invention because it has the GST-encoding DNA inserted upstream from the multiple cloning sites (MCSs) thereof. It should be noted, however, that

the present invention can be embodied using any other optionally chosen expression plasmid.

Next, an appropriate host cell (*e.g.*, *Escherichia coli*) is transformed with the thus-obtained expression vector, followed by transformant cultivation using an appropriate medium to yield the desired fusion protein. The resultant fusion protein can be purified by adsorbing a cell-free extract thereof to a column of Glutathione Sepharose 4B (produced by Pharmacia), washing the column with phosphate-buffered saline (hereinafter PBS) containing 1% Triton X-100, then with a DTT-containing Tris-HCl buffer, and eluting with a glutathione-containing buffer. As stated later, high-purity GST-NS5Bt was successfully obtained in large amounts. The NS5Bt protein of the non-fusion type can be separated from the fusion protein as necessary.

The present invention is hereinafter described in more detail by means of the following examples, which are not to be construed as limiting the scope thereof. The plasmids, various restriction enzymes, T4 DNA ligase and other enzymes used in the Examples below were purchased from commercial sources, and were used as directed by the respective suppliers. Also, DNA cloning, plasmid construction, host transformation, transformant cultivation and enzyme recovery from cultures were conducted in accordance with methods known to those skilled in the art or methods described in the literature.

Reference Example 1: Western blotting

(1) Preparation of antiserum against NS5B

The antiserum was induced in a rabbit by subcutaneously injecting 200 μg of purified bacterial hexahistidine-labeled NS5Bt in Freund's complete adjuvant (Sigma Chemicals Co., Ltd.) to the rabbit. The IgG fraction of the antiserum was purified using a column of

Protein A Sepharose, as directed by the supplier (Pharmacia LKB Co., Ltd.). Both the antiserum and the purified IgG fraction were used for western blotting.

(2) Western blotting

A protein sample (prepared from *Escherichia coli* or mammalian cell extract (lysate)) was suspended in an SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycol, 5% 2-mercaptoethanol), heated for 5 minutes, fractionated by 10% SDS-PAGE, and electrically transferred onto a nitrocellulose membrane (Schleicher & Schuell Co., Ltd.) in transfer buffer (192 mM glycine, 25 mM Tris and 20% methanol). Next, using the anti-NS5B, the various recombinant NS5B proteins expressed were detected by the conventional method of western blotting. The bands were observed using ECL (trade name: ECL Western Blotting Detection Reagent Kit, Catalog Code RPN2106P, Amersham Co., Ltd.), as directed by the supplier (Amersham Co., Ltd.).

Reference Example 2: Determination of polymerase activity

For NS5B, GST-NS5Bt, rNS5Bt, and samples containing them, RNA-dependent RNA polymerase activity was, as a rule, determined by the method described below. Other polymerase activities (reverse transcriptase activity, DNA-dependent RNA polymerase activity, DNA-dependent DNA polymerase activity) and terminal transferase activity were also determined at the same time.

The above activities were assessed on the basis of [α-^{32}P]UMP or [α-^{32}P]dTMP incorporation in accordance with methods described in the literature (Behrens, S.E. *et al.*, EMBO J. 15:12-22, 1996; De Francesco, R. *et al.*, Methods Enzymol. 275:58-67, 1996; Lama, J. *et al.*, J. Biol. Chem. 270:14430-14438, 1995).

A standard reaction (10 μl) was carried out in a buffer containing

20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20 U RNase inhibitor (Wako Pure Chemical Industries), 2 μ Ci [α-^{32}P]UTP (800 Ci/mmol, Amersham Co., Ltd.), 10 μ M UTP, 10 μ g/ml poly(A) and oligo$(U)_{14}$. To this reaction mixture, purified NS5Bt was added, followed by incubation at 25℃ for 2 hours, after which the sample was transferred onto a DE81 filter (Whatman Co., Ltd.) to stop the reaction. The filter was thoroughly washed with 0.5 M Na_2HPO_4 (pH 7.0), rinsed with 70% ethanol, and dried in air, after which the radioactivity bound to the filter was finally determined using a liquid scintillation counter.

2) RdRP activity was determined using both oligo$(U)_{14}$ and oligo(dT) as primers. RT activity was determined using poly(A), oligo(dT) and [α-^{32}P]dTTP as a template, primer, and substrate, respectively. RNA polymerase activity was determined using poly(dA), oligo(U), and [α-^{32}P]UTP. To assess the effects of *Escherichia coli*-derived polymerase, rifampicin and actinomycin D (Sigma Co., Ltd.) were dissolved in ethanol and added to the reaction system.

3) RNA-dependent RNA polymerase (RdRP) assay

RdRP activity was determined using a total of 40 μl of a reaction mixture [20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20 U RNase inhibitor (Wako Pure Chemical Industries), 50 μ g/ml actinomycin D (Sigma Co., Ltd.), 5 μ Ci [α-^{32}P]UTP and 0.5 mM of each of the remaining NTPs (ATP, CTP and GTP)] and 10 μg/ml RNA template. The restricted nucleotide concentration was adjusted to 10 μ M. After the purified NS5Bt sample was added, the reaction mixture was incubated at 30℃ for 2 hours, after which an equal amount of 2 x proteinase K buffer (300 mM NaCl, 100 mM Tris-HCl (pH 7.5), 1% SDS) was added and the mixture was digested with 50 μg of proteinase K (Boehringer Mannheim Co., Ltd.) for 30 minutes to stop the reaction.

The RNA product was extracted with phenol-chloroform (1:1), subjected to ethanol precipitation, and analyzed by 8 M urea-8% PAGE. After electrophoresis, the gel was dried, exposed to an imaging plate, and analyzed using the BAS 1000 Bioimage analyzer (Fuji Co., Ltd.).

Example 1: Production of recombinant GST-NS5Bt and NS5Bt

1. Construction of the plasmid pGENKS

The expression plasmid pGENKS for a recombinant protein of GST fusion type was prepared as follows: pGENK1 (Murakami, S. *et al.*, J. Biol. Chem. 269:15118-15123, 1994; Yi, M.-K. *et al.*, Virology 231:119-129, 1997) was digested with *Eco*RI and *Bam*HI to yield the pGENK1 *Eco*RI/*Bam*HI vector, into which the following synthetic oligonucleotides encoding multiple cloning sites were inserted.

GAATTCGAGC TCCGGTACCC CCGGGTCGAC GACGGATCC SSFor (SEQ ID NO:4)

GGATCCGTCG ACCCCGGGGG TACCGGAGCT C CGAATC SSRev (SEQ ID NO:5)

The above synthetic oligonucleotides were annealed at 65℃ in the presence of NaCl, digested with *Eco*RI-*Bam*HI, extracted with phenol-chloroform, and precipitated with ethanol, after which they were dissolved in a Tris-EDTA solution (hereinafter TE) and ligated to the above vector using Ligation High (Toyobo), as directed by the supplier. The *Escherichia coli* XL1-Blue strain was then transformed with this vector by the conventional method to yield the plasmid pGENKS.

The plasmid pGENKS encodes the consensus sequence of protein kinase A, thrombin cleavage site and multiple cloning sites (*Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sal*I and *Bam*HI) of protein kinase A, upstream of which the GST-encoding DNA sequence is present. The restriction map and construction scheme for this plasmid pGENKS are shown in FIG. 2 and

FIG. 3, respectively. In FIG. 3, the site of cleavage of the GST-NS5Bt fusion protein by thrombin is underlined.

2. Transformation of *Escherichia coli*

Escherichia coli was transformed by an ordinary method of molecular biology. The plasmid DNA or ligation reaction liquid was added to 60 μl of competent *Escherichia coli*, followed by incubation on ice for 30 minutes and subsequent incubation at 42℃ for about 1 minute and on ice for 2 minutes, after which an SOC solution was added; after 45 minutes of culture at 37℃, the culture was plated onto an LB plate containing 100 μg/ml ampicillin and cultured at 37℃ for about 12 to 20 hours to yield a transformant.

3. Construction of the GST-NS5Bt expression vector pGENKS/NS5Bt

(1) Preparation of NS5B DNA

An HCV JK1 subgenomic cDNA containing NS5B (Honda, M. *et al.*, Arch Virol. 128:163-169, 1993) was subcloned by PCR using the following pair of primers, which have an initiation codon for protein synthesis, artificial *Sac*I and *Sal*I restriction sites.

GGGAGCTCCA TGTCGATGTCT TACACGTGGAC A (NS5B For) (SEQ ID NO:6)

GGGTCGACCC GGTTGGGGAGC AGGTAGATGCC (NS5B Rev) (SEQ ID NO:7)

Using these two primers and Taq polymerase (TaKaRa Ex Taq; produced by Takara Shuzo Co., Ltd.), PCR was conducted by the procedures shown below, as directed by the supplier. Specifically, a series of treatment comprising (94℃, 1 minute; 58℃, 1 minute; 72℃, 2 minutes) was repeated in 30 cycles.

By this operation, an about 1.7 kb NS5B cDNA was amplified by

PCR. This PCR product was subjected to low-melting agarose gel electrophoresis, the DNA band was cut out, and the DNA was recovered by ordinary phenol, chloroform and ethanol precipitation. The recovered cDNA was treated with restriction enzymes *Sac*I and *Sal*I (Takara Shuzo Co., Ltd.), treated with phenol and chloroform, precipitated with ethanol, and purified, after which it was used in the next step.

(2) Construction of pGENKS/NS5B

A plasmid expressing full-length NS5B was first constructed. pGENKS was treated with restriction enzymes *Sac*I and *Sal*I to yield the pGENKS *Sac*I/*Sal*I vector, to which the NS5B cDNA *Sac*I/*Sal*I fragment obtained in (1) above was ligated using the T4 DNA ligase kit Ligation High (Toyobo), as directed by the supplier, followed by transformation of *Escherichia coli*, to yield pGENKS/NS5B.

(3) Construction of pGENKS/NS5Bt

NS5Bt, having 21 C-terminal amino acids deleted, was subjected to a PCR reaction in the same manner as above, using NS5BFor and the following primer (NS5BtRev):

GGGTCGACGC GGGGTCGGGCA CGAGACAGGCT (NS5BtRev) (SEQ ID NO:8

with pGENKS/NS5B as obtained in (2) above as a template, to yield an NS5Bt cDNA, which was treated in the same manner as above to yield pGENKS/NS5Bt.

(3) Expression of GST-NS5Bt using *Escherichia coli* transformant and its purification

An *Escherichia coli* strain was transformed by the method described in 2 above and cultured by the method described below, after which the expression product was purified.

Samples were taken at various stages of purification and subjected to SDS-10% PAGE to separate the protein, and the protein concentration was determined. At the same time, each sample was analyzed by the method of western blotting (anti-NS5B and anti-GST antibodies were used) described in Reference Example 1. The protein concentration was determined by Bradford's method or by Coomassie staining (CBB) comparing with bovine serum albumin (BSA) as standard. In addition, polymerase activity of samples taken at various stages of purification was assessed on the basis of UMP incorporation, as described in Reference Example 2 and (7) below. The results of these determinations are shown in FIG. 4 and Table 1.

1) Preparation of bacteria expressing the proteins

One colony of an *Escherichia coli* strain (BL21pLysS) resulting from transformation with the pGENKS/NS5Bt plasmid was suspended in 10 ml of an LB medium containing 100 μg/ml ampicillin, and precultured at 30℃ overnight. This culture was diluted with 1 l of an LB medium containing 100 μg/ml ampicillin, and cultured at 30℃ until the OD_{600} value reached 0.6 to 0.7; to each culture, 0.4 mM isopropyl-β-D-thiogalactopyranoside (INCORPORATION) was added for overnight induction.

One liter of this culture was centrifuged, *Escherichia coli* cells were harvested and once washed with phosphate-buffered saline (PBS). Next, this pellet was suspended in 32 ml of PBS containing 1 mM dithiothreitol (DTT) and 1% Triton X-100 (hereinafter buffer A).

2) Sonication

The suspension was sonicated on ice until the viscosity was lost, followed by centrifugation at 15,000 g for 20 minutes. Supernatant 1 (S1) and the pellet were separated; the supernatant (S1) was kept

standing on ice. The pellet was suspended in 32 ml of buffer A containing 1.0 M NaCl; the resulting suspension was sonicated in the same manner as above and centrifuged; the supernatant was collected (S2) and mixed with S1 above; the resulting mixture was adjusted to an NaCl concentration of 0.33 M with buffer A to yield supernatant 3 (S3).

3) Purification with DEAE Sephacel

S3 was passed through a column of DEAE Sephacel, previously equilibrated with buffer A, to yield an effluent fraction.

4) Purification with a column of glutathione Sepharose 4B

The fraction obtained in 3) above was mixed with 1 ml of glutathione Sepharose 4B beads (Pharmacia Biotech Co., Ltd.), previously equilibrated in buffer A, and kept standing at 4℃ for 1 hour to absorb the protein to the beads. Next, these beads were thoroughly washed with buffer A, then with 50 mM Tris-HCl (pH 8.0) containing 1 mM DTT, eluted with 4 ml of an elution buffer [50 mM Tris-HCl (pH 8.0), 10 mM glutathione, 10 mM DTT and 0.1% Triton X-100], after which it was eluted with 4 ml of an elution buffer containing 500 mM NaCl (FIG. 4A, lane 4).

5) Purification with a column of heparin Sepharose

Next, after being adjusted to an NaCl concentration of 150 mM, the eluate was applied to a column of heparin Sepharose (Pharmacia Biotech Co., Ltd.), previously equilibrated with an elution buffer containing 150 mM NaCl. After being washed with an LG buffer containing 150 mM NaCl, the column was eluted with an LG buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 20% glycerol, 0.5% Triton X-100] on an NaCl concentration gradient from 100 mM to 1 M to separate individual fractions.

Analysis of the eluate by 10% SDS-PAGE revealed that GST-

NS5Bt was eluted over a wide NaCl concentration range from 500 mM to 900 mM.

6) Purification with a column of poly(U) Sepharose

The fractions obtained in 5) above were combined and adjusted to an NaCl concentration of 150 mM with LG buffer. Next, this solution was applied to a column of poly(U) Sepharose (Pharmacia Biotech Co., Ltd.), previously equilibrated with an LG buffer containing 150 mM NaCl.

After being washed with an LG buffer containing 150 mM NaCl, the column was eluted with an LG buffer containing 200 mM to 1 M NaCl. Analysis by 10% SDS-PAGE in the same manner as in 5) above revealed that GST-NS5Bt was eluted over an NaCl concentration range from 500 mM to 700 mM. These fractions were combined and dialyzed against an LG buffer containing 150 mM NaCl (FIG. 4A, lane 5).

(5) Purification of non-fusion type rNS5Bt

The GST-NS5Bt bound to glutathione resin, prepared in (4), 4) above, was treated with thrombin to cleave it at the artificial consensus sequence (underlined in FIG. 3) in the junction with GST. Specifically, glutathione beads (glutathione Sepharose 4B beads) bound with the GST-NS5Bt were thoroughly washed with a thrombin cleavage buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$, 1% Triton X-100). Next, the beads were treated at 4℃ overnight using a thrombin cleavage buffer containing thrombin (50 U) (Pharmacia Biotech Co., Ltd.), to release the GST-fused NS5Bt protein from the GST carrier. After the beads were centrifuged, the supernatant, which contained the NS5Bt protein, was dialyzed against an LG buffer containing 150 mM NaCl; the sample obtained was assayed for protein concentration and analyzed by western blotting (FIG. 4A, lane 6). Polymerase activity was

also determined.

(6) Effect of purification

Changes in purity over the time course covering different purification processes are shown in FIG. 4, and those in polymerase activity in Table 1. In FIG. 4, panel A shows the results of sample separation by SDS-10% PAGE and subsequent staining by Coomassie staining (CBB). Lane 1 shows the results for the whole cell extract (lysate); lane 2 shows the results for the centrifugal supernatant of the sonication product; lane 3 shows the results for the cell-free extract after passage through Sephacel; lane 4 shows the results for the eluate from the column of glutathione Sepharose 4B; lane 5 shows the results for the eluate from the column of poly(U) Sepharose; lane 6 shows the results for non-fusion type rNS5Bt after thrombin treatment. Panels B and C shows the results of western blotting using the anti-NS5B antibody and the anti-GST antibody, respectively, for the samples taken at these 6 purification stages.

As shown on lane 5 of FIG. 4, a 95 kDa recombinant GST-NS5Bt protein was obtained at a purity exceeding 90%.

Also, analysis of this 95 kDa band by western blotting demonstrated that it was specifically recognized by both rabbit anti-NS5Bt IgG and anti-GST IgG (FIG. 4B and C, lane 5). The purified GST-NS5Bt protein corresponding to the 95 kDa protein was analyzed by western blotting using serum obtained from a chronic hepatitis patient infected with HCV 1b or a healthy volunteer. In FIG. 5, lanes 1 and 2 are the purified GST-NS5Bt and the GST protein as control, respectively. It can be seen from the FIG. 5 that GST-NS5Bt of about 95 kDa was recognized specifically by serum of a chronic hepatitis patient infected with HCV 1b (indicated by arrow). These results shown in FIGs. 4 and

5 demonstrated that the resultant protein is a recombinant GST-NS5Bt.

On lane 6 of FIG. 4A, a double band is seen at a position corresponding to an about 63 kDa protein of the non-bound fraction of the thrombin digest. In western blotting, this band was specifically recognized by anti-NS5Bt IgG antibody but not by anti-GST IgG, confirming its identity as a non-fusion type rNS5Bt protein (FIG. 4B and C).

Also, from Table 1, it is seen that UMP incorporation by the purified GST-NS5Bt was detected, with a relative activity at least 10,000 times that of the whole cell lysate. The thrombin-cleaved non-fusion NS5Bt exhibited UMP incorporation activity, although at a lower level than with the fusion protein.

The finally purified fusion protein (GST-NS5Bt) and non-fusion protein (NS5Bt) were thoroughly dialyzed against an LG buffer containing 150 mM NaCl and stored at -80℃.

(7) Detection of polymerase activity

The polymerase activity of the purified GST-NS5Bt obtained in (6) above, which exceeded 90% in purity, was analyzed in accordance with the method described in Reference Example 2. Specifically, incorporation of [α -^{32}P]UMP or [α -^{32}P]dTMP as a template was determined using the purified GST-NS5Bt (90 ng). The results are shown in FIG. 6. In the figure, panel A shows the substrate specificity determined with poly(A) or poly(dA) as a template and oligo$(U)_{14}$ or oligo(dT) as a primer. Panel B shows the time courses of reactions at 25℃ and 37℃. Panel C shows the relationship between the amount of GST-NS5Bt and UMP incorporation.

From FIG. 6, it is evident that UMP incorporation increases proportionally with the amount of GST-NS5Bt.

Poly(A) dependent UMP incorporation in the assay was much higher in the case of the oligo(U) primer than in the case of the oligo(dT) primer (about 2 times) (FIG. 6A). Also, the purified GST-NS5Bt did not exhibit reverse transcriptase activity, DNA-dependent RNA polymerase or DNA polymerase activity under the conditions tested (FIG. 6A).

Incorporation was sustained at 25℃ for at least 4 hours. At 37℃, however, the incorporation rate was slower, becoming plateau after 2 hours (FIG. 6B).

Table 1 Purification of HCV RNA polymerase

Purification Step	Concentration (μg/ml)	Incorporation* (cpm)	Specific Activity**	Relative Activity***
Whole cell lysate	18000	1319±230	0.0009	1
Suppressant after sonication	6000	1485±82	0.003	3.4
DEAE passage	5900	1558±53	0.0003	3.6
Glutathione elution	350	12462±2281	0.44	485
Poly(U) column	90	67740±1413	9.41	10271
Thrombin cleavage	280	47280±3771	2.11	2344

*: [α-^{32}P]UMP incorporation into reaction system

**: pmole/μg/hour

***: Relative to incorporation by total cell lysate.

Also, the effects of various factors on polymerase activity were examined. The results are shown in FIGS. 7 and 8 and Tables 2 and 3.

As shown in Table 2, UMP incorporation is not observed in the case of GST alone. Moreover, in the absence of the primer or template, UMP incorporation is not observed either, demonstrating that the

NS5Bt protein does not possess terminal transferase activity (Table 2). This property differs from the activity of NS5B expressed in insect cells.

Also, NS5Bt was not inhibited in the presence of 20 μg/ml rifampicin or 50 μg/ml actinomycin D, nor was inhibited even when their concentrations were increased to 200 μg/ml and 500 μg/ml, respectively (Table 2).

Table 2 Summary of RdRP activity in GST-NS5Bt

	UMP Incorporation* (cpm)	Activity (%)
Perfect	99448±2170	100
- Primer[oligo(U)]	1124±88	1.12
- Template[poly(A)]	2249±1142	2.43
- Primer, template	1521±299	1.52
Rifampicin (20 μg/ml)*	25175±4628	105
Actinomycin D (20 μg/ml)*	23894±5467	100
- Mg^{2+}	738±94	0.74
GST	423±14	0.42
- Protein	1241±242	1.24

*: The control contained an equal amount of ethanol.

The RdRP activity of purified GST-NS5Bt, based on UMP incorporation, was examined under various conditions. The reaction was carried out using GST-NS5Bt (30 to 45 ng). Optimum pH was determined by incubation at 25℃ for 2 hours in a sodium phosphate buffer. The results are shown in FIGS. 7 and 8.

In FIG. 7, panel A shows UMP incorporation at different pH levels; panel B shows UMP incorporation (or uptake) at different temperatures; panel C shows UMP incorporation at different KCl concentrations; in FIG. 8, panel D shows UMP incorporation at different Mg^{2+} ion concentrations; panel E shows UMP incorporation at different Zn^{2+}

concentrations (0, 10, 25 and 50 μM). The results are summarized as follows:

1) Optimum pH for UMP incorporation ranges widely near the neutral point (FIG. 7A).

2) UMP incorporation is most effective at 30℃ (FIG. 7B).

3) UMP incorporation is inhibited at KCl concentrations not lower than 100 mM KCl, strictly depending on the Mg^{2+} ion, the optimum Mg^{2+} concentration ranging from 2.5 to 5 mM (FIG. 8D).

4) Other divalent ions, such as Zn^{2+}, have no effects on UMP incorporation (FIG. 8E).

Also, assessment of the effects of detergents (surfactants) demonstrated that ionic detergents (0.01% Sarkosyl or SDS) completely inhibited the activity, while non-ionic detergents (*e.g.*, Triton X-100, Nonidet P-40, Tween 20 and CHAPS) at concentrations up to 0.1% had almost no effects (Table 3).

Table 3 Effects of detergents on activity

Detergent*	0.01 %**	0.1 %**	1.0 %**
Triton X-100	90.8	89.2	61.8
Nonidet P-40	97.2	98.7	63.1
Tween 20	104.5	121.0	118.9
Tauro DOC	81.2	12.6	12.6
SDS	4.6	4.1	5.0
Sarcosyl	28.8	7.5	3.6
CHAPS***	83.2	85.6	100.8

*: Activity from standard reaction (detergent-free) (25755 cpm) taken as 100%.

**: Final concentration (v/v)

***: CHAPS concentrations 0.1 mM, 1 mM and 10 mM, respectively.

(8) RNA synthesis assay with HCV RNA as template and primer

Following the UMP incorporation experiment of (7) above, in which a synthetic template and primer were used, the potential of GST-NS5Bt in using HCV RNA as a template and primer was investigated. The reaction was carried out at 30℃ for 2 hours, using the same reaction mixture as that used in the above-described RNA polymerase assay.

The HCV 3' UTR (site to function as replication initiation site) was divided into 3 regions, as shown in FIG. 9A. 5BBg is a region of NS5B covering the *Bgl*II site to the 3' UTR; poly(U) is a poly(U) stretch; and 3'X is a highly conserved sequence at the 3'-terminus of the HCV genomic RNA; 3'X, which has been suggested as playing an important role in viral replication (Kolykhalov, A.A. *et al.*, J. Virol. 70:3363-3371, 1996; Tanaka, T. *et al.*, J. Virol. 70:3307-3312, 1996), is contained. These various HCV subgenomic RNAs were synthesized by *in vitro* transcription and used for RNA synthesis assay (see FIG. 9A).

1) Preparation of templates

To prepare an RNA template, various plasmids (pGEM3zf(+)/NS5BBg, pGEM3zf(+)/poly(U), and pGEM3zf(+)/3'X) were constructed and subjected to *in vitro* transcription to yield the RNA template. These plasmids were constructed by PCR using the synthetic oligonucleotide primers shown below.

pGEM3zf(+)/NS5BBg was constructed by PCR using the primers:
GCGGATCCAG ATCTACGGGGC CACTTA (5BBgFor) (SEQ ID NO:9)
GCGAATTCAA GACAAAGGGAA TGGCCTAT (5BBgRev) (SEQ ID NO:10)
(these primers have a synthetic *Bam*HI site and *Eco*RI site, respectively), with pGEM3zf(+)/HCV JK-1 (containing HCV JK-1 cDNA between the *Eco*RI and *Bam*HI sites) as a template.

pGEM3zf(+)/poly(U) was constructed by annealing the oligonucleotides:

GCGATTCGA AGACTTCCCTT TTTTTTGTT TTTTTTTTTT CTTTTTT TTTT TTTCTTTTTT TTCCTTTTTT TTTTTTCT (polyUFor) (SEQ ID NO:11)

GCGGATCCGA AGACGCCACCA AAGAAGGAAAA GGGAAAAAAAA AAAACAAAGAA GAAAAAAAAA AAAAAGGAAAAA AAAGA (polyURev) (SEQ ID NO:12)

(these oligonucleotides have a synthetic *Eco*RI site, *Bam*HI site and *Bbs*I site, respectively), followed by PCR cloning, to yield a fragment containing the poly(U) stretch of HCV, and inserting this DNA fragment into the pGEM3zf(+) (Promega Co., Ltd.) *Eco*RI and *Bam*HI vector.

pGEM3zf(+)/3'X was constructed by annealing the set of oligonucleotides:

GCGAATTCGA AGACTTGGTGG CTCCATCTTAG CCCTAGTCACG GCTAGCTGTGA AAGGTCCGTG AGCCGCATGAC TGCAG (3' XFor) (SEQ ID NO:13)

GCGGATCCCT TAAGACATGAT CTGCAGAGAGG CCAGTATCAGC ACTCTCTGCAG TCATGCGGCT CAC (3'X Rev) (SEQ ID NO:14)

(these oligonucleotides have a synthetic *Eco*RI site, *Bbs*I site, *Afl*II site and *Bam*HI site, respectively), followed by PCR cloning to yield a fragment containing 3'X (Kolykhalov, A.A. *et al.*, J. Virol. 70:3363-3371, 1996; Tanaka, T. *et al.*, J. Virol. 70:3307-3312, 1996), and inserting the DNA fragment having the *Eco*RI and *Bam*HI sites into the pGEM3zf(+) (Promega Co., Ltd.) *Eco*RI and *Bam*HI vector.

All these constructs were analyzed using Taq sequencing kits and a DNA sequencer (374A, Applied Biosystems Co., Ltd.) to confirm their sequences.

2) Preparation of templates

The above plasmids pGEM3zf(+)/5BBg, pGEM3zf(+)/poly(U) and pGEM3zf(+)/3'X were linearized by digestion with *Bam*HI or *Eco*RI. Separately, pNKFLAG as constructed by the method described in Test Example 1 below was digested with *Bgl*II and used to prepare a control RNA.

In vitro transcription using these templates was conducted using T7 RNA polymerase or SP6 RNA polymerase (Promega Co., Ltd.), as directed by the supplier.

After incubation, each DNA template was digested with RNase-free DNase. The resulting RNA product was extracted with phenol-chloroform (1:1) and passed through a column of Sephadex G-50 (Pharmacia Biotech Co., Ltd.) to remove free nucleotides, after which it was subjected to ethanol precipitation. After the RNA concentration was determined by spectrophotometer and adjusted to 1 μg/μl with RNase-free distilled water, the sample was stored at -20℃.

RNA sample quality was confirmed by electrophoresis using MOPS-denatured gel or urea-denatured polyacrylamide gel.

In accordance with the description in Reference Example 2, 3), RNA-dependent RNA polymerase (RdRP) assay was conducted. After proteinase K treatment and subsequent extraction with phenol-chloroform, the RNA product was subjected to ethanol precipitation and finally separated and purified by 8M urea-containing 8% PAGE. The sizes of the respective RNAs are as shown in FIG. 9A.

The results are shown in FIG. 9B. New radiolabeled bands slightly greater than those for the starting RNA were detected (FIG. 9B, lanes 1, 3 and 4).

RNA synthesis by the GST-NS5Bt of the present invention requires all the 4 ribonucleotides as substrates; when UTP or CTP was

used alone, no incorporation was detected. The HCV RNA and control RNA acted as templates and primers (FIG. 9). The RNA synthesis capability using the 3'X of the HCV 3'UTR was slightly lower than other RNAs (FIG. 9, compare lanes 1 and 4 and 3); the poly(U) stretch of the HCV 3' UTR as such did not act as a template and primer (FIG. 9, lane 2). These results demonstrate that RNA is used as a template and primer, and that GST-NS5Bt possesses no terminal transferase activity, as shown by the UMP incorporation assay.

From the findings in (7) and (8) above, it is seen that the recombinant GST-NS5Bt and recombinant NS5Bt expressed by the *Escherichia coli* transformant of the present invention are both active soluble proteins possessing characters common to all RdRPs (template and primer requirements, Mg^{2+} dependency, optimum reaction conditions, etc.). The GST-NS5Bt and NS5Bt of the present invention also differ from the conventional recombinant NS5B expressed in insect cells in terms of activity in that they exhibit no terminal transferase activity.

Test Example 1 Relationship between activity and amino acid sequence in GST-NS5Bt and NS5Bt

Various variants having a variation in amino acid sequence were prepared to assess the effects of variations on RdRP activity.

The NS5Bt variants designed were a substitutional variant (NS5Bt-m1) with a variation in the HCV NS5B GDD motif, which is highly conserved among all RdRPs, a variant (NS5Bt-m2) with a variation in YRHRAR and a variant (NS5Bt-m3) with a variation in CGYRRCR (see FIG. 1).

These variants are substitutional variants resulting from substitution of GDD by VDD (amino acid Nos. 317-317 in SEQ ID NO:1

(amino acid Nos. 2736-2738 in the HCV protein, the same applies below)), substitution of YRHRAR by AAAAAA (500-505 (2919-2924)), and substitution of CGYRRCR by AAAAAA (274-280 (2693-2699)).

The DNAs encoding these substitutional variants were prepared by the PCR mutagenesis method based on overlapping extension using NS5BFor and NS5BtRev, both primers designed for mutagenesis (Yi, M.-K. *et al.*, Virology 231:119-129, 1997).

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A substitutional variant of NS5B with a variation in the C-terminal region (NS5B-m4) was obtained by PCR with NS5BFor and NS5Bm4Rev, having the sequence shown below, as primers.

GCGGATCCTC ACCGGTTGGGG AGCAGGTAGAT GCCTACCCCGG AGAAGGTAGGA GTAGGCACCA CAT (NS5Bm4Rev) (SEQ ID NO:15)

This NS5Bm4Rev, having a synthetic *Bam*HI site, was used as a 3' primer.

In NS5B-m4, both the L and V at amino acid Nos. 579 and 582 have been converted to P (see FIG. 1).

The activities of these variants were determined; the results are shown in Table 4.

Table 4 RdRP activities of GST-NS5Bt mutants

Protein	Incorporation (cpm)	Activity (%)
GST-NS5Bt (20 ng)	22085	100
GST-NS5Bt-m1 (20 ng)	359	1.5
(40 ng)	248	1.1
GST-NS5Bt-m2 (20 ng)	70	0.3
(40 ng)	172	0.7
GST-NS5Bt-m3 (20 ng)	124	0.5
(40 ng)	113	0.5

Of the GST-NS5Bt variant proteins recovered from *Escherichia coli* transformants, NS5Bt-m1 did not show RdRP activity at all (see Table 4), suggesting that the GST-NS5Bt or NS5Bt protein requires the GDD motif for their RNA synthesis activity. Substitutional variants with a variation in a cluster consisting of a relatively large number of base residues, namely NS5Bt-m2 and NS5Bt-m3, also did not show RdRP activity at all, and did not inhibit the RdRP activity of the GST-NS5Bt obtained in Example 1 (Table 4). These findings demonstrate that the RdRP activity obtained is derived from GST-NS5Bt, and that the important GDD motif is required for the activity.

Test Example 2 Subcellular localization of GST-NS5Bt in mammalian cells

The effects of deletion of 21 C-terminal amino acids in NS5B on the subcellular localization of NS5B in mammalian cells were assessed in transient expression systems of mammalian cells (cultured hepatoma cell line HLE cells, HepG2 cells, non-hepatoma cell line COS1 cells).

To facilitate NS5B detection, expression plasmids encoding fusion proteins of green fluorescent protein (GFP) and full-length NS5B, NS5Bt and NS5B-m4, respectively, were constructed. With these plasmids and a plasmid encoding GFP alone, mammalian cell line HLE cells were transformed, and the subcellular localization of the protein expressed was examined.

(1) Construction of plasmids for expression of fusion proteins with GFP in mammalian cells

These plasmids were prepared from pSG5UTPL (Lin, Y. *et al.*, J. Biol. Chem. 272:7132-7139, 1997; Murakami, S. *et al.*, J. Biol. Chem. 269:15118-15123, 1994).

cDNAs of green fluorescent protein (GFP) were prepared by PCR using the primers shown below with phGFP-S56T (Clontech Co., Ltd.) as a template.

AAGATATCGC GGCCGCATGGT GAGCAAGGGCG AG (GFPNotFor) (SEQ ID NO:16)

AAGGATCCGA ATTCTTGTACA GCTCGTCCAT (GFPEcoRev) (SEQ ID NO:17)

These primers have a synthetic *Eco*RV site, *Not*I site and *Eco*RI site, respectively. The DNA fragment having a synthetic *Eco*RV site and *Bam*HI site was treated with *Eco*RV and *Bam*HI; the *Eco*RI site of pSG5UTPL was blunted using the Klenow fragment, followed by insertion into a *Bam*HI-digested vector, to yield pGFP. Using this pGFP vector, another mammalian expression vector, pNKFLAG, was constructed.

A sequence having a region associated with translation initiation, and encoding the FLAG-tag (label) epitope sequence, was obtained from pFLAGHis/p53 (obtained from R. Roeder) by the PCR method described below. This DNA fragment was inserted into the pGFP *Not*I and *Bam*HI vector by PCR using the following set of primers:

ATGCGGCCGCCACCATGGACTACAAAGACGAT (NKFLAGFor) (SEQ ID NO:18)

CGGGATCCTCAGTCTGAGTCAGGCCCTTCT (p53Rev) (SEQ ID NO:19)

(these primers each have a synthetic *Not*I site, a consensus translation initiation site, and a *Bam*HI site), to yield pNKFLAG/p53.

The plasmid pNKFLAG was constructed by substituting the p53 insert with multiple cloning sites using an *Eco*RI site and a *Bam*HI site, as described above.

To the *Eco*RI and *Bam*HI sites of these plasmids pNKFLAG and

pGFP, the NS5B DNA was inserted by the conventional method to construct a GFP-NS5B expression plasmid (pGFP/NS5B) or a FLAG-labeled NS5B expression plasmid (pNKFLAG/NS5B). Similarly, the DNA encoding NS5Bt and NS5B-m4 was inserted to construct the respective expression plasmids.

(2) Expression in mammalian cells

About 1 x 10^5 HLE cells were plated onto a slide glass and placed on a Quadriperm microscope slide culture well (Heraeus Co., Ltd.); on the following day, these cells were transfected with the GFP-NS5B expression plasmid (pGFP/NS5B) or the FLAG-labeled NS5B expression plasmid (pNKFLAG/NS5B). After rinsing with PBS, the cells were fixed with PBS containing 1.5% para-formaldehyde, followed by post-fixation with 100% cold methanol for 5 minutes. The cells were then dried in air at -25℃ and stored at -80℃. The GFP-fusion protein was detected by contrast staining with 0.0005% Evans Blue in PBS. Samples with FLAG-labeled protein expression were blocked with 0.5% BSA in PBS and subjected to overnight staining with the anti-FLAG M2 antibody diluted (1:330) with PBS containing 0.5% BSA.

Immunological staining was achieved by contrast staining with Evans Blue using absorbed rabbit anti-mouse IgG, biotinylated goat anti-rabbit IgG and streptoavidin-FITC (Amersham Co., Ltd.) by the standard technique.

The results were observed under a BX-50 fluorescent microscope (Olympus Incorporation Co., Ltd.) equipped with an NIBA filter and WIB filter, and visualized by digital printing (Pictrography 3000, Fuji Co., Ltd.) The expression level of the same GFP-fusion protein in HLE cells was immunologically detected by the western blotting method described in Reference Example 1 using anti-NS5B IgG and anti-GFP-IgG

(Clontech Co., Ltd.). Similar experiments were conducted for other NS5B variants.

The results are shown in FIG. 10. In the figure, panels (A) through (D) show the results for (A) full-length NS5B, (B) GFP-NS5Bt, (C) GFP-NS5B-m4, and (D) GFP alone, respectively.

From FIG. 10, it is seen that the GFP-NS5B protein was distributed mainly around the cytoplasmic perinuclear membrane, with some dispersion in cytoplasm, and that it was localized as diffused in both cytoplasm and the nucleus in the case of GFP alone (FIG. 10A). Also, it is seen that the GFP-NS5Bt protein was accumulated mainly in the nucleus, with its subcellular localization significantly affected by cleavage of the C-terminal region (FIG. 10B). In FIG. 10, the fluorescent intranuclear signal of GFP-NS5Bt is observed as several clusters having large irregular spherical shapes.

Also, GFP-NS5B-m4, which has 2 substitutional variations designed to inhibit the function of the C-terminal anchor region of NS5B, was also localized in the nucleus, with some distribution in cytoplasm (FIG. 10C). Similar results were obtained from other mammalian cells, *i.e.*, COS1 cells or HepG2 cells. These results suggest that the C-terminal region of 21 amino acids of NS5B plays an important role as an anchor for NS5B protein immobilization on the membrane in its subcellular localization.